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Identification of 25-hydroxyvitamin D_3 1α-hydroxylase gene expression in macrophages

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Identification of 25-hydroxyvitamin D_3 1α-hydroxylase gene expression in macrophages.

Background. The 25-hydroxyvitamin D_3 1α-hydroxylase (1α-hydroxylase) is almost exclusively expressed in the kidney. However, 1α-hydroxylase activities have been observed in some extrarenal tissues, including inflammatory cells of the monocyte/macrophage lineage. In sarcoidosis, macrophage 1α-hydroxylase causes overproduction of 1,25-$(OH)_2D_3$, resulting in hypercalcemia. In this study, we investigated the regulation of macrophage 1α-hydroxylase at a molecular level.

Methods. We used the human monocytic cell line THP-1, which can be differentiated into macrophage-like cells by treatment with phorbol ester. The expression of 1α-hydroxylase in THP-1 cells was examined by Northern blotting and immunoblotting using an antibody raised against a synthetic peptide corresponding to the 14 C-terminal amino acids of 1α-hydroxylase. We investigated the regulation of 1α-hydroxylase mRNA expression by RNase protection assay.

Results. Northern blot and immunoblot analyses confirmed the expression of 1α-hydroxylase in THP-1 cells at the mRNA and protein levels. Although parathyroid hormone and calcitonin, known stimulators of renal 1α-hydroxylase, did not affect the expression of 1α-hydroxylase mRNA, 8-Br-cAMP (5×10^{-4} mol/L) increased the expression of 1α-hydroxylase mRNA in THP-1 cells (198 ± 9%). 1,25-$(OH)_2D_3$, known as a suppressor of renal 1α-hydroxylase, did not affect the expression of 1α-hydroxylase mRNA. By contrast, 1,25-$(OH)_2D_3$ markedly increased the expression of 25-hydroxyvitamin D_3 24-hydroxylase mRNA. Interferon-γ (2000 IU/mL) increased the expression of 1α-hydroxylase mRNA in differentiated THP-1 cells (922 ± 25%).

Conclusions. The present results suggest that 1α-hydroxylase activity in macrophages is mediated by the same enzyme as in kidney. Interferon-γ treatment increases macrophage 1α-hydroxylase levels via directly increasing gene expression of this enzyme.

The active form of vitamin D_3, 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$], is an important regulator of bone and mineral metabolism [1]. In addition, this hormone has multiple effects on cells of the immune system [2]. 1,25-$(OH)_2D_3$ induces differentiation of several human cell lines of the monocytic lineage and of normal human hematopoietic cells. The hormone modulates various functions of monocytes/macrophages, such as chemotaxis, hydrogen peroxide production, antigen presentation, and secretion of prostaglandin E_2, and influences the activity of T and B lymphocytes [3–8].

In the formation of 1,25-$(OH)_2D_3$, 25-hydroxyvitamin D_3 1α-hydroxylase (1α-hydroxylase) is the key enzyme in the determination of 1,25-$(OH)_2D_3$ levels. 1α-Hydroxylase is almost exclusively expressed in the kidney; however, 1α-hydroxylase activities have been observed in some extra-renal tissues. Placenta, bone cells, keratinocytes, and macrophages from the alveolar space, peritoneal cavity, and bone marrow have been reported to synthesize 1,25-$(OH)_2D_3$ in vitro [9–15]. The local production of the active metabolite of vitamin D in target organs suggests an autocrine/paracrine role for 1,25-$(OH)_2D_3$ in these tissues. Extra-renal 1,25-$(OH)_2D_3$ production does not contribute to systemic 1,25-$(OH)_2D_3$ concentrations under physiological conditions. However, in pregnancy [16] or in some pathological states, extrarenal 1α-hydroxylase contributes to circulating 1,25-$(OH)_2D_3$. Hypercalcemia occurs in about 10% of the patients with sarcoidosis [17]. Abnormal calcium metabolism is due to dysregulated production of 1,25-$(OH)_2D_3$ by activated 1α-hydroxylase in macrophages trapped in pulmonary alveoli and granulomatous inflammation [12, 18].

The metabolic control of 1α-hydroxylase activity in macrophages is different from that in renal epithelial cells. Renal 1α-hydroxylase activity is under stringent regulation by parathyroid hormone (PTH), calcitonin, calcium, phosphorus, and 1,25-$(OH)_2D_3$ itself [1]. In contrast, macrophage 1α-hydroxylase is relatively immune to stimulation by calcium or PTH or to feedback inhibition by 1,25-$(OH)_2D_3$, but is sensitive to stimulation by interferon-γ [13, 19, 20]. These different mechanisms of regulation between renal and macrophage 1α-hydroxylase raise the

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question of whether the macrophage 1α-hydroxylase is the same enzyme as the renal 1α-hydroxylase.

Human monocytic THP-1 cells provide a valuable model for study of the regulation of 1α-hydroxylase in macrophages. After treatment with phorbol esters, THP-1 cells differentiate into macrophage-like cells, which mimic native monocyte-derived macrophages in several respects [21]. Phorbol ester-differentiated THP-1 cells constitutively expressed 1α-hydroxylase activity, and 1,25-$(OH)_2D_3$ production increased 30-fold in response to interferon-γ [22].

We recently cloned a 1α-hydroxylase cDNA from human kidney [23]. This enabled us to investigate the macrophage 1α-hydroxylase at a molecular level. In this study, we examined 1α-hydroxylase expression in THP-1 cells at the mRNA and protein levels and the effects of PTH, calcitonin, cAMP, 1,25-$(OH)_2D_3$, and interferon-γ on the expression of 1α-hydroxylase mRNA in these cells.

METHODS

Reagents

8-Br-cAMP, phorbol 12-myristate 13-acetate (PMA), and actinomycin D were purchased from Sigma Chemical (St. Louis, MO, USA). Human PTH (1-34) and salmon calcitonin was purchased from Bachem Feinchemikalien AG (Bubendorf, Switzerland). 1,25-$(OH)_2D_3$ was purchased from Duphar (Weesp, The Netherlands). Recombinant human interferon-γ was purchased from Genzyme Corporation (Cambridge, MA, USA).

Cell culture

Human monocytic THP-1 cells (American Type Culture Collection TIB202; ATCC, Rockville, MD, USA) were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. THP-1 cells were differentiated by exposure to 160 nmol/L PMA for 24 hours and were then washed twice with phosphate-buffered saline (PBS) and once with serum-free media. In all experiments, serum was removed from the media 12 hours before the addition of reagents. THP-1 cells were incubated with reagents in RPMI 1640 containing 0.1% fatty acid-free bovine serum albumin for 24 hours. After medium was removed, the cells were washed with PBS, and RNA or protein was prepared.

Northern blotting

Poly $(A)^+$ RNA was prepared using Micro-Fast Track kit (Invitrogen, Carlsbad, CA, USA) from THP-1 cells. Approximately 2 μg of poly $(A)^+$ RNA were denatured with glyoxal and dimethyl sulfoxide, separated on a 1% agarose gel, and blotted onto a nylon membrane. The blot was hybridized for 18 hours in ExpressHyb hybridization solution (Clontech, Palo Alto, CA, USA) at 68°C, with a [^{32}P]-labeled probe prepared from a full-length human 1α-hydroxylase cDNA. A final high-stringency wash was performed at 50°C in 0.1 × standard saline citrate (SSC), 0.1% sodium dodecyl sulfate (SDS), and the blot was exposed to a BAS Imaging Plate (Fuji Photo Film, Tokyo, Japan). The blot was normalized by probing with β-actin. In Northern blot analysis of vitamin D receptor (VDR), we used a [^{32}P]-labeled probe prepared from a human VDR cDNA [24].

Antibodies

Rabbit polyclonal antibody was raised against a synthetic peptide corresponding to the 14 C-terminal amino acids of 1α-hydroxylase (LVPERSINLQFLDR). A cysteine residue was attached to the N-terminus of the peptide to introduce SH residue for coupling. The 15-amino acid CLVPERSINLQFLDR was synthesized by the f-moc method. The synthetic peptide conjugated with keyhole limpet hemocyanin by sulfo-MBS method was used to immunize two New Zealand white rabbits with Freund's complete adjuvant (first injection) or incomplete adjuvant (from second injection). After five injections, a sufficient increase of the antibody titer was confirmed by enzyme-linked immunosorbent assay (ELISA), and serum was collected. Specific antibodies were prepared from the antiserum by affinity column chromatography using the antigen peptide coupled to FMP-activated Cellulofine (Seikagaku Kogyo, Tokyo, Japan).

Immunoblotting

THP-1 cells were washed with PBS and treated with 10% (volume/volume) trichloroacetic acid for 15 minutes on ice. The precipitated proteins were collected by centrifugation, dissolved in 80 μL of 9 mol/L urea containing 2% (volume/volume) Triton X-100 and 1% (weight/volume) dithiothreitol, and disrupted by sonication. Then 20 μL of loading buffer containing 10% (weight/volume) lithium dodecyl sulfate and Bromophenol blue were added to the solubilized protein. These samples, corresponding to approximately 2×10^5 cells of THP-1 cells were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblotting. SDS-PAGE was performed as described by Laemmli [25] on 7.5% gels. Proteins were transferred onto a Hybond ECL nitrocellulose filter (Amersham Pharmacia Biotech AB, Uppsala, Sweden) and probed with anti–1α-hydroxylase antibody. Bound antibodies were visualized by an ECL Western blotting system (Amersham Pharmacia Biotech AB). COS-7 cells transfected with human 1α-hydroxylase cDNA were used as a positive control. To determine antigen specificity, the antibody solutions were preabsorbed with the antigen peptide.

Riboprobes

DNA fragments corresponding to bases 1073 to 1354 of human 1α-hydroxylase cDNA [23], bases 1049 to 1421

of human 25-hydroxyvitamin D_3 24-hydroxylase cDNA [26], and bases 748 to 894 of human β-actin cDNA [27] were amplified from reverse-transcribed human kidney cDNA and subcloned into plasmid pT7Blue-T vector (Novagen, Madison, WI, USA). All constructs were confirmed by sequence analysis. Plasmids were linearized with EcoR I, and antisense RNA probes were labeled with [α-^{32}P]UTP using T7 RNA polymerase. The synthesized full-length cRNAs were gel purified from a polyacrylamide gel and then subjected to RNase protection assay. The sizes of the protected fragments was 282 bp for the human 1α-hydroxylase, 347 bp for the human 24-hydroxylase, and 147 bp for the human β-actin.

RNase protection assay

Total RNA was extracted from THP-1 cells using ISOGEN (Nippon Gene, Tokyo, Japan). The RNase protection assay was carried out according to the manufacturer's protocol to an RPA II kit (Ambion, Austin, TX, USA). For each experiment, 10 μg of total RNA and three [^{32}P]-labeled riboprobes were mixed and coprecipitated. After centrifugation, each sample was suspended in 20 μL of hybridization buffer, heated to 95°C for three minutes, and then hybridized at 45°C for 12 to 18 hours. The hybridization mixture was digested with 200 μL of RNase A (1.25 U/mL) and RNase T1 (50 U/mL) at 37°C for 30 minutes, followed by precipitation. The protected fragments were resolved on a 5% polyacrylamide gel containing 8 mol/L urea. The gel was transferred to chromatography paper and exposed to a BAS Imaging Plate. Band intensities were quantitated using a FUJIX BIO-Imaging Analyzer BAS 2000 (Fuji Photo Film) and normalized to the signal generated by the constitutively expressed β-actin gene. Each experiment was conducted at least four times.

RT-PCR and Southern blotting

To examine the effect of actinomycin D on the interferon-γ–induced 1α-hydroxylase mRNA expression, we employed reverse transcription-polymerase chain reaction (RT-PCR) and Southern blotting for mRNA quantitation. THP-1 cells were incubated with 2000 IU/mL of interferon-γ in the presence or absence of 10 μg/mL of actinomycin D for six hours. Messenger RNA for 1α-hydroxylase and β-actin were amplified by PCR using the specific primer sets (5′-GGAGTGGACACGGTGTCCAA-3′ and 5′-ACACAGAGTGACCAGCGTATT-3′ for 1α-hydroxylase, and 5′-TGGAGAAGAGCTACGAGCTG-3′ and 5′-ACTTCATGATGGAGTTGAAGG-3′ for β-actin). For the linear relationship between the amount of RNA and PCR product, we employed 28 cycles for 1α-hydroxylase and 18 cycles for β-actin. Analysis of PCR products was performed by agarose gel electrophoresis followed by Southern blotting and then hybridized to an internal oligonucleotide (5′-GTCCAGACAGCACTCCACTCAGAGA-3′ for 1α-hydroxylase, and 5′-ACCATTGGCAATGAGCGGTTCCGCT-3′ for β-actin) that lies within the target sequence between the two original primers. The band intensities were measured by densitometric scanning and were normalized with the band intensities of β actin. Three independent experiments were performed.

Statistical analysis

The results were expressed as a percentage of the control values or the maximum stimulation. Data are expressed as mean ± SEM. Statistical significance was evaluated by Student's *t*-test or one-way analysis of variance (ANOVA) with a Scheffe's post hoc test, as appropriate. Differences were considered significant when $P < 0.05$.

RESULTS

Northern blot analysis

We recently showed that 1α-hydroxylase mRNA is almost exclusively expressed in the kidney in humans [23]. We examined renal 1α-hydroxylase mRNA expression in human monocytic THP-1 cells by Northern blot analysis using a 1α-hydroxylase cDNA cloned from human kidney as a probe. 1α-Hydroxylase gene transcripts were not detected in normal THP-1 cells (Fig. 1, lane 1). When THP-1 cells were differentiated by incubation with 160 nmol/L PMA for 24 hours, followed by stimulation by 2000 IU/mL interferon-γ, a 2.5 kb band consistent with 1α-hydroxylase mRNA was detected (Fig. 1, lane 2).

Immunoblot analysis

We generated a polyclonal antibody specific for 1α-hydroxylase to examine 1α-hydroxylase protein expression. This antibody was produced against a synthetic peptide corresponding to the 14 C-terminal amino acids of 1α-hydroxylase (LVPERSINLQFLDR), which is unique for 1α-hydroxylase. The specificity of the antibody was tested by immunoblot analysis with homogenate prepared from COS-7 cells transfected with human 1α-hydroxylase cDNA. The antibody recognized a single major band at approximately 55 kD (Fig. 2, lane 1). The band was abolished by pretreatment of the antibody with the antigen peptide (Fig. 2, lane 6). The differentiated THP-1 cells did not show any band (Fig. 2, lanes 2 and 3). When differentiated THP-1 cells were incubated with 2000 IU/mL of interferon-γ for 24 hours, a 55 kD band consistent with 1α-hydroxylase protein was detected (Fig. 2, lanes 4 and 5). The band was abolished by pretreatment of the antibody with the antigen peptide (Fig. 2, lanes 9 and 10). We could not see any band after incubation with 10^{-8} mol/L $1,25(OH)_2D_3$ for 24 hours (Fig. 2, lane 13). This finding supports that our antibody did not cross-react with 25-hydroxyvitamin D_3 24-hydroxylase protein.

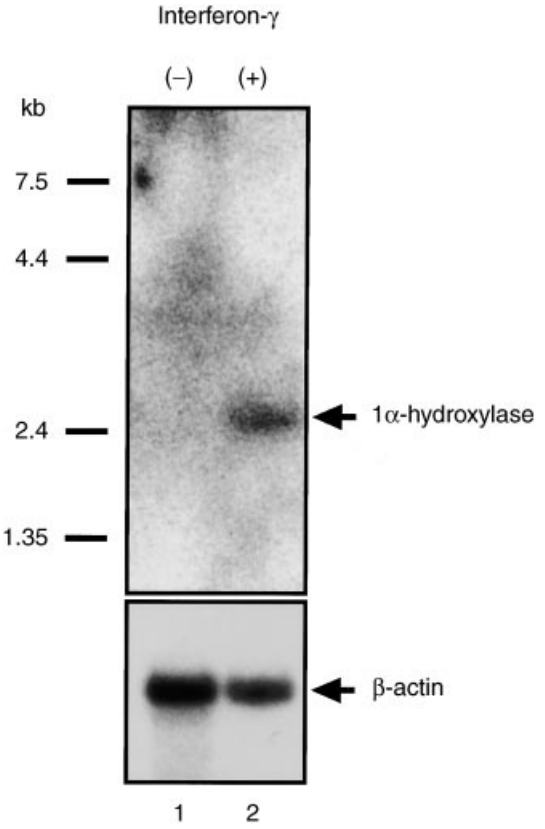

Fig. 1. Northern blot analysis of 1α-hydroxylase expression. Two microgram of poly(A)$^+$ RNA were separated on 1% agarose gel and probed with 1α-hydroxylase cDNA (upper panel) or β-actin (lower panel). Lane 1, THP-1 cells, and lane 2, differentiated THP-1 cells incubated with 2000 IU/mL interferon-γ for 24 hours. The relative position of size markers is indicated on the left.

RNase protection assay

1α-Hydroxylase is expressed at relatively low levels, and thus, we used RNase protection assay to measure more sensitively the regulation of 1α-hydroxylase mRNA expression in THP-1 cells. Riboprobes for 1α-hydroxylase, 24-hydroxylase, and β-actin were transcribed from the DNA templates corresponding to bases 1073 to 1354 of human 1α-hydroxylase cDNA [23], bases 1049 to 1421 of human 24-hydroxylase cDNA [26], and bases 748 to 894 of human β-actin cDNA [27], respectively. The three probes detected signals for 1α-hydroxylase mRNA, 24-hydroxylase mRNA, and β-actin mRNA.

Effect of differentiation

First, 1α-hydroxylase mRNA expression along with differentiation of THP-1 cells was examined. The 1α-hydroxylase mRNA levels were low in undifferentiated THP-1 cells. Upon differentiation by incubation with 160 nmol/L PMA for 24 hours, 1α-hydroxylase mRNA expression was significantly increased (303 ± 9%, $N = 4$, $P < 0.05$; Fig. 3A).

Effect of PTH, calcitonin, and cAMP

Parathyroid hormone and calcitonin are known stimulators of renal 1α-hydroxylase. However, 10^{-7} mol/L PTH did not stimulate 1α-hydroxylase mRNA expression in differentiated THP-1 cells, and 10^{-7} mol/L calcitonin also had no significant effect (Fig. 3B). The effects of PTH and calcitonin are exerted in part through the protein kinase A (PKA) pathway. We found that 5×10^{-4} mol/L of 8-Br-cAMP, a PKA activator, increased 1α-hydroxylase mRNA levels (198 ± 9%, $N = 4$, $P < 0.05$; Fig. 3B). We could not detect PTH receptor or calcitonin receptor expression in THP-1 cell by RT-PCR (data not shown).

Effect of 1,25-$(OH)_2D_3$

The effect of 1,25-$(OH)_2D_3$, an important negative regulator of renal 1α-hydroxylase, on 1α-hydroxylase mRNA expression in undifferentiated and differentiated THP-1 cells was examined. Even at a concentration of 10^{-8} mol/L, 1,25-$(OH)_2D_3$ did not affect 1α-hydroxylase mRNA expression (Fig. 4).

24-Hydroxylase is known to be responsible for 1,25-$(OH)_2D_3$ inactivation. Under basal conditions, 24-hydroxylase mRNA levels were undetectable. Incubation with 10^{-8} mol/L 1,25-$(OH)_2D_3$ dramatically induced 24-hydroxylase mRNA expression (Fig. 5). The responsiveness of 24-hydroxylase gene expression to 1,25-$(OH)_2D_3$ demonstrates the presence of VDR in THP-1 cells. In addition, Northern blot analysis for VDR showed that the differentiated THP-1 cells expressed VDR receptor mRNA and that treatment with 2000 IU/mL of interferon-γ did not affect the mRNA expression of VDR (Fig. 6).

Effect of interferon-γ

Interferon-γ is a macrophage-specific potent stimulator of 1α-hydroxylase. Figure 7 shows the effects of incubation of undifferentiated and differentiated THP-1 cells with increasing amounts of interferon-γ on the expression of 1α-hydroxylase mRNA. Interferon-γ exerted a dose-dependent stimulatory effect on the expression of 1α-hydroxylase mRNA in THP-1 cells. At a concentration of 2000 IU/mL, mRNA expression was increased (196 ± 8%, $N = 4$, $P < 0.05$; Fig. 7A). In differentiated THP-1 cells, the stimulatory effect of interferon-γ was more obvious; it increased 1α-hydroxylase expression at a concentration of 2000 IU/mL (922 ± 25%, $N = 4$, $P < 0.05$; Fig. 7B). The effect of interferon-γ observed in RNase protection assay was comparable with the results obtained by Northern blot analysis and immunoblot analysis. In a time course study, the stimulatory effect of interferon-γ was observed at 6 hours, and maximal effect was observed at 24 hours (data not shown).

To determine whether the regulation of the 1α-hydroxylase mRNA expression by interferon-γ occurs at a transcriptional level, we examined the effect of the RNA

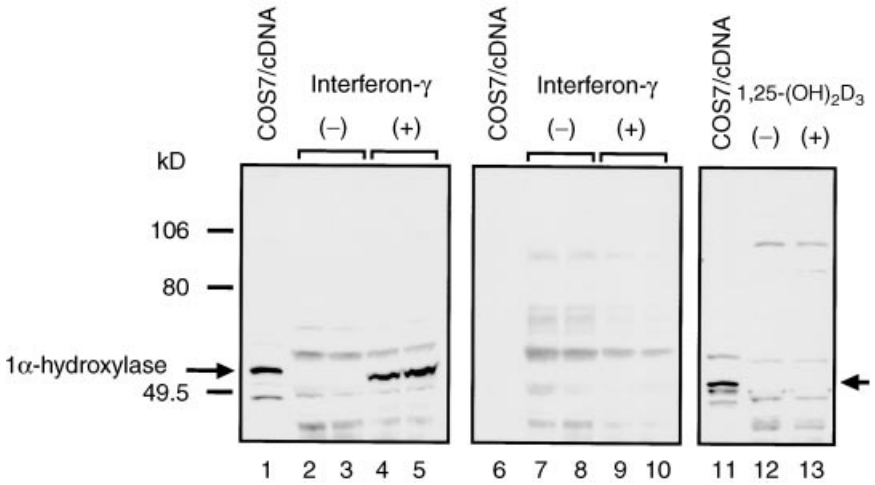

Fig. 2. Immunoblot analysis of 1α-hydroxylase in THP-1 cells. Lanes 1, 6, and 11, homogenates from COS-7 cells transfected with 1α-hydroxylase cDNA. Lanes 2, 3, 7, 8 and 12, homogenates from differentiated THP-1 cells. Lanes 4, 5, 9, and 10, homogenates from differentiated THP-1 cells incubated with 2000 IU/mL interferon-γ for 24 hours. Lane 13, homogenate from differentiated THP-1 cells incubated with 10^{-8} mol/L 1,25-$(OH)_2D_3$ for 24 hours. Immunoblot analysis probed with antibody against 1α-hydroxylase (lanes 1 through 5 and 11 through 13) or with the antibody preabsorbed with the antigen peptide (lanes 6 through 10). The positions of molecular weight markers are shown on the left.

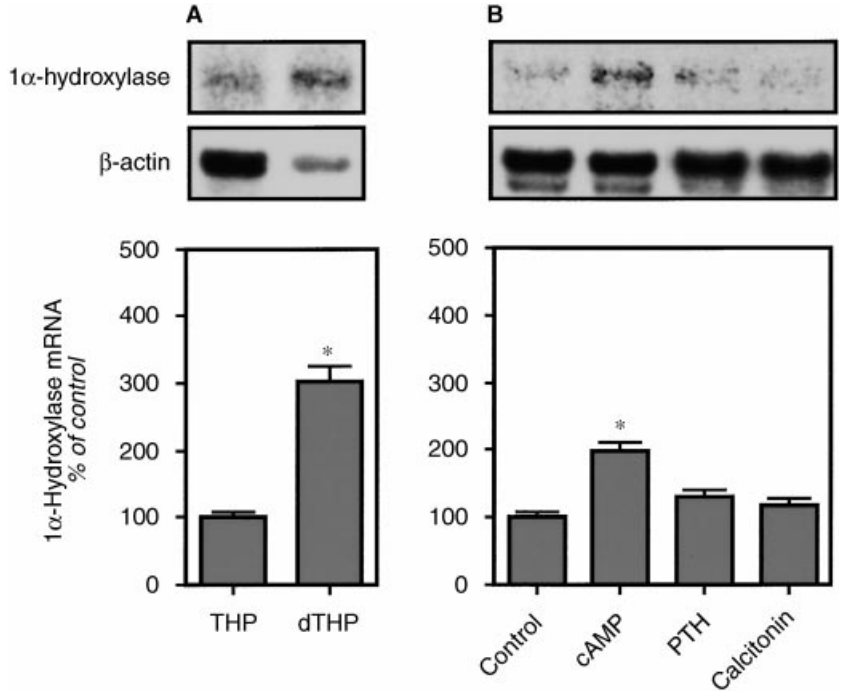

Fig. 3. (*A*) Expression of 1α-hydroxylase mRNA along with differentiation of THP-1 cells. Total RNA was extracted from undifferentiated THP-1 cells or differentiated THP-1 cells (dTHP) by incubation for 24 hours with PMA (160 nmol/L). (*B*) Effect of PTH, calcitonin, and cAMP on 1α-hydroxylase mRNA expression. Total RNA was extracted from differentiated THP-1 cells incubated for 24 hours with vehicle (lane 1), 8-Br-cAMP (5×10^{-4} mol/L; lane 2), PTH (10^{-7} mol/L; lane 3), or calcitonin (10^{-7} mol/L; lane 4). In the upper panels in A and B, autoradiograms of RNase protection assay show protected fragments of 1α-hydroxylase (282 bp) and β-actin (147 bp) mRNA. The lower panels in A and B show quantitative analysis of 1α-hydroxylase gene expression. Data were normalized using β-actin mRNA and expressed as a percentage of the control values. $*P < 0.05$ vs. control ($N = 4$).

synthesis inhibitor actinomycin D on the induction of 1α-hydroxylase gene expression by interferon-γ. Quantitative RT-PCR showed the effect of interferon-γ on 1α-hydroxylase mRNA expression was abolished in the presence of 10 μg/mL of actinomycin D (Fig. 8). This indicates that the effect of interferon-γ on 1α-hydroxylase mRNA expression is, at least in part, a transcriptional event.

Effect of combination of 1,25-$(OH)_2D_3$ and interferon-γ

Dusso et al demonstrated that interferon-γ and 1,25-$(OH)_2D_3$ had a cross-talk effect on the expression of hydroxylases [22]. We observed that 2000 IU/mL of interferon-γ markedly suppressed 24-hydroxylase mRNA expression induced by 10^{-8} mol/L 1,25-$(OH)_2D_3$ in undifferentiated cells ($92.3 \pm 4.1\%$, $N = 4$, $P < 0.05$; Fig. 5A). On the other hand, in differentiated THP-1 cells, 2000 IU/mL of interferon-γ suppressed it by only $53.5 \pm 2.4\%$ ($N = 4$, $P < 0.05$; Fig. 5B). This indicates that the inhibitory effect of interferon-γ on 1,25-$(OH)_2D_3$ action decreases as monocytes differentiate to macrophages. We examined the combined effect of interferon-γ and 1,25-$(OH)_2D_3$ on 1α-hydroxylase mRNA expression. Incubation with 1,25-$(OH)_2D_3$ (10^{-8} mol/L) and interferon-γ (2000 IU/mL) together was not significantly different to that of interferon-γ (2000 IU/mL) alone (Fig. 9).

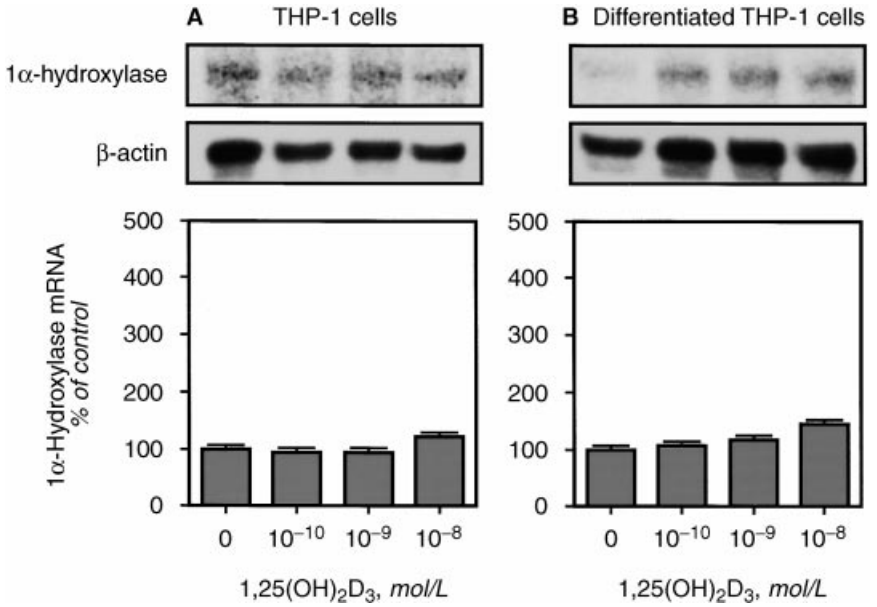

Fig. 4. Effect of 1,25-$(OH)_2D_3$ on 1α-hydroxylase mRNA expression. Total RNA was extracted from undifferentiated (*A*) or differentiated (*B*) THP-1 cells incubated for 24 hours with 1,25-$(OH)_2D_3$ (0, 10^{-10}, 10^{-9}, or 10^{-8} mol/L). In the upper panels in A and B, autoradiograms of RNase protection assay show protected fragments of 1α-hydroxylase (282 bp) and β-actin (147 bp) mRNA. The lower panels in A and B show quantitative analysis of 1α-hydroxylase gene expression. Data were normalized using β-actin mRNA and were expressed as a percentage of the control values. $^*P < 0.05$ vs. control ($N = 4$).

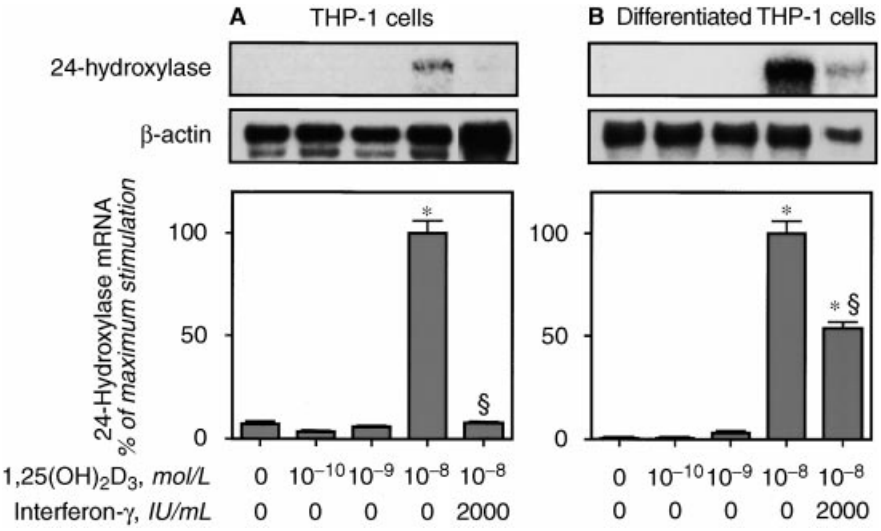

Fig. 5. Effect of 1,25-$(OH)_2D_3$ on 24-hydroxylase mRNA expression. Total RNA was extracted from undifferentiated (*A*) or differentiated (*B*) THP-1 cells incubated for 24 hours with 1,25-$(OH)_2D_3$ (0, 10^{-10}, 10^{-9}, or 10^{-8} mol/L) or 1,25-$(OH)_2D_3$ (10^{-8} mol/L) together with interferon-γ (2000 IU/mL). In the upper panels in A and B, autoradiograms of RNase protection assay show protected fragments of 24-hydroxylase (347 bp) and β-actin (147 bp) mRNA. The lower panels in A and B show quantitative analysis of 24-hydroxylase gene expression. Data were normalized using β-actin mRNA and expressed as a percentage of the values at the maximum stimulation. $^*P < 0.05$ vs. control; §$P < 0.05$ vs. 1,25-$(OH)_2D_3$ (10^{-8} mol/L) alone ($N = 4$).

DISCUSSION

Human monocytic THP-1 cells provide the best current model of mature monocytes [21]. Upon differentiation along the monocytic lineage by treatment with phorbol ester, THP-1 cells acquire macrophage-like properties [28] and 1α-hydroxylase activity [22]. In this study, Northern blot analysis demonstrated the expression of renal 1α-hydroxylase gene transcripts in THP-1 cells. Immunoblot analysis using 1α-hydroxylase–specific antibody showed the expression of 1α-hydroxylase protein in THP-1 cells. Since the mRNA and protein are up-regulated by incubation with interferon-γ, there is less possibility of cross-reaction with other hydroxylases in the detection of mRNA and protein. Thus, we can conclude that the macrophage 1α-hydroxylase is mediated by the same gene product as the renal 1α-hydroxylase.

THP-1 cells lacked the responsiveness for PTH or calcitonin, which are known to be positive regulators for renal 1α-hydroxylase. We can attribute this to an absence of their receptors in THP-1 cells. It is known that the effect of PTH is mediated through PKA, whereas the effect of calcitonin is mediated through both PKA and protein kinase C (PKC). In the present study, 8-Br-cAMP, a PKA activator, induced 1α-hydroxylase mRNA expression in THP-1 cells. Recently, we showed that the effect of calcitonin on 1α-hydroxylase mRNA expression is mediated through the PKC-signaling pathway, rather than through the PKA-signaling pathway [29]. Murayama et al also

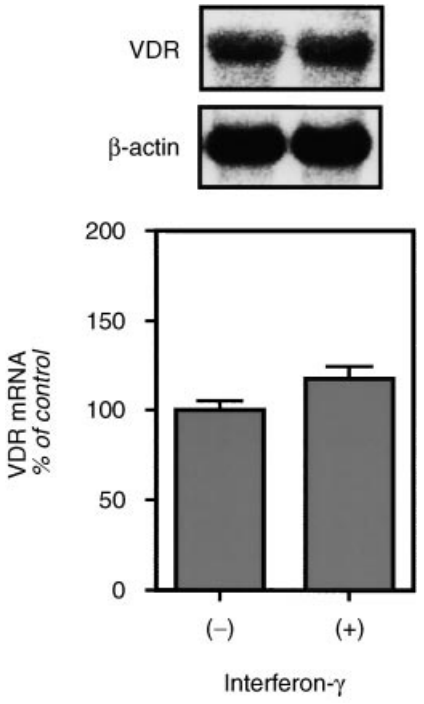

Fig. 6. Vitamin D receptor (VDR) mRNA expression in differentiated THP-1 cells. Total RNA was extracted from differentiated THP-1 cells incubated with vehicle or interferon-γ (2000 IU/mL) for 24 hours. In the upper panels, autoradiograms of Northern blot analysis show the bands of VDR and β-actin mRNA. The lower panel shows quantitative analysis of VDR gene expression. Data were normalized using β-actin mRNA and were expressed as a percentage of the control values.

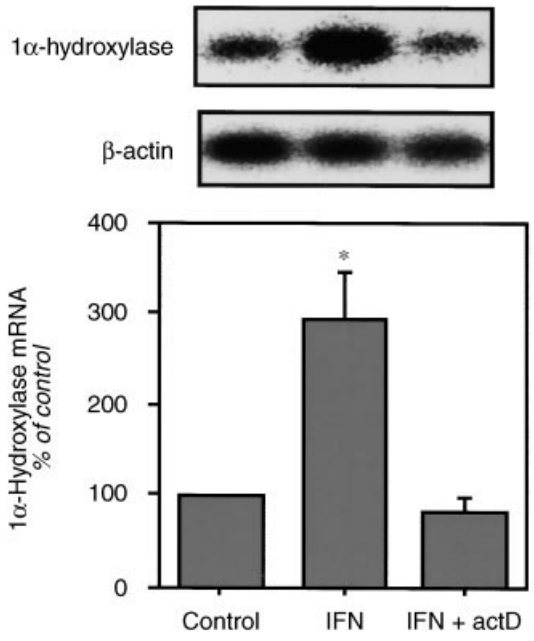

Fig. 8. Effect of actinomycin D on interferon-γ-induced 1α-hydroxylase mRNA expression. Total RNA was extracted from differentiated THP-1 cells incubated for six hours with vehicle, interferon-γ (2000 IU/mL), or interferon-γ (2000 IU/mL) and actinomycin D (10 μg/mL). In the upper panels, autoradiograms of RT-PCR/Southern blot analysis show the bands of 1α-hydroxylase and β-actin mRNA. The lower panel shows quantitative analysis of 1α-hydroxylase gene expression. Data were normalized using β-actin mRNA and were expressed as a percentage of the control values. $*P < 0.05$ vs. control ($N = 3$).

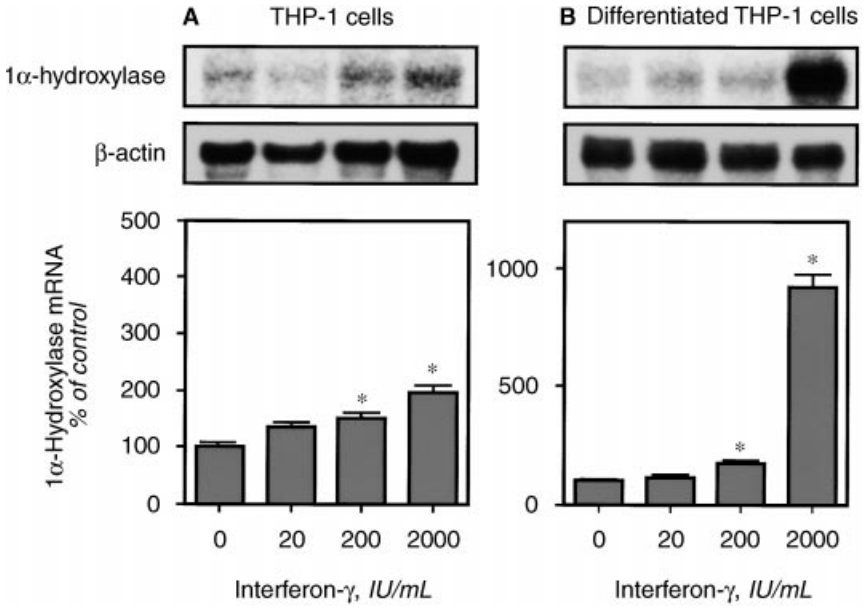

Fig. 7. Effect of interferon-γ on 1α-hydroxylase mRNA expression. Total RNA was extracted from undifferentiated (*A*) or differentiated (*B*) THP-1 cells incubated for 24 hours with interferon-γ (0, 20, 200, or 2000 IU/mL). In the upper panels in A and B, autoradiograms of RNase protection assay show protected fragments of 1α-hydroxylase (282 bp) and β-actin (147 bp) mRNA. The lower panels in A and B show quantitative analysis of 1α-hydroxylase gene expression. Data were normalized using β-actin mRNA and were expressed as a percentage of the control values. $*P < 0.05$ vs. control ($N = 4$).

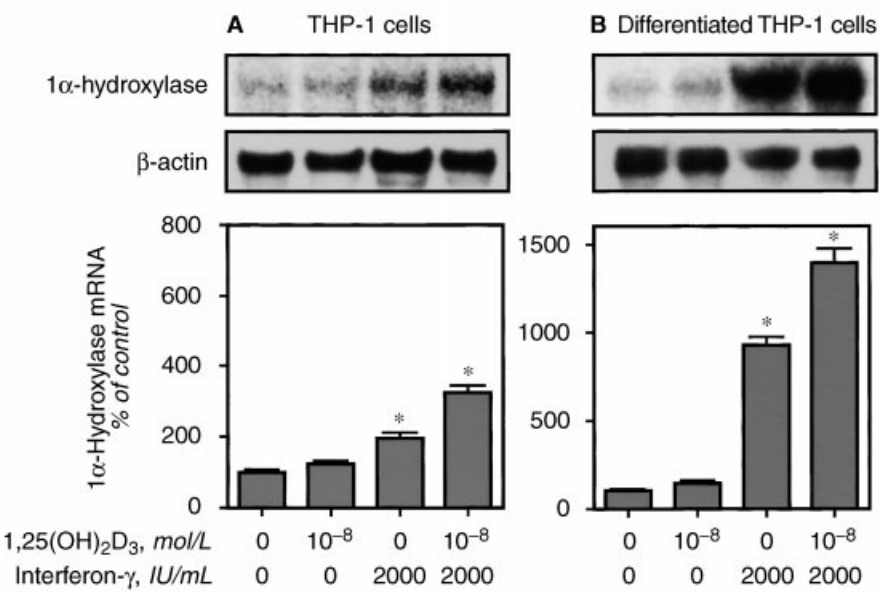

Fig. 9. Effect of 1,25-$(OH)_2D_3$ on 1α-hydroxylase mRNA expression in interferon-γ–stimulated THP-1 cells. Total RNA was extracted from undifferentiated (*A*) or differentiated (*B*) THP-1 cells incubated for 24 hours with vehicle, interferon-γ (2000 IU/mL), 1,25-$(OH)_2D_3$ (10^{-8} mol/L), or interferon-γ (2000 IU/mL) together with 1,25-$(OH)_2D_3$ (10^{-8} mol/L). In the upper panels in A and B, autoradiograms of RNase protection assay show protected fragments of 1α-hydroxylase (282 bp) and β-actin (147 bp) mRNA. The lower panels in A and B show quantitative analysis of 1α-hydroxylase gene expression. Data were normalized using β-actin mRNA and were expressed as a percentage of the control values. $^*P < 0.05$ vs. control ($N = 4$).

reported PTH and calcitonin enhance 1α-hydroxylase mRNA through the different signaling pathways [30].

Our study shows that interferon-γ stimulates 1α-hydroxylase activity at the level of gene expression. The main intracellular signaling pathway after binding of interferon-γ to its specific receptor is via the JAK-STAT pathway. In response to interferon-γ, Jak1 and Jak2 are activated, followed by activation of Stat1α [31]. Activated Stat1α forms Stat1α/Stat1α homodimers that bind a gamma-activating sequence (GAS; TTCNNNAA consensus sequence) on the promoter region of the gene, and the transcription is activated [32]. We have cloned the 5′-flanking region of the 1α-hydroxylase gene. While no GAS was found, some homologous sequences were identified in the 1.5 kb promoter region of 1α-hydroxylase. We are planning to investigate the effect of interferon-γ on the promoter activity of 1α-hydroxylase gene using a reporter gene system.

1,25-$(OH)_2D_3$ itself suppresses the enzyme activity of 1α-hydroxylase in renal cells. In macrophages, the inhibitory effect of the 1,25-$(OH)_2D_3$ was less pronounced than that in renal cells. A supranormal concentration (75 nmol/L) of 1,25-$(OH)_2D_3$ reduced 1,25-$(OH)_2D_3$ synthesis by only 20% in sarcoid macrophages [19], suggesting an apparent lack of control in the 1α-hydroxylation reaction by 1,25-$(OH)_2D_3$ in sarcoid macrophages. In the present study, we failed to demonstrate any inhibitory effect of 1,25-$(OH)_2D_3$ on 1α-hydroxylase mRNA expression in THP-1 cells. Even a concentration of 10^{-7} mol/L 1,25-$(OH)_2D_3$ did not suppress expression (data not shown). The addition of 1,25-$(OH)_2D_3$ to interferon-γ–stimulated THP-1 cells had no effect on 1α-hydroxylase mRNA levels. In contrast, 1,25-$(OH)_2D_3$ markedly increased 24-hydroxylase mRNA expression in THP-1 cells. This confirms that the 1,25-$(OH)_2D_3$ used in our experiments was active and that THP-1 cells expressed VDR. In addition, we confirmed VDR mRNA expression in THP-1 cells by Northern blot analysis. The expression of VDR mRNA was not affected by the treatment of interferon-γ (2000 IU/mL). Dusso et al also demonstrated that 1,25-$(OH)_2D_3$-VDR binding could not account for the dramatic loss of 1,25-$(OH)_2D_3$ responsiveness in interferon-γ–treated THP-1 cells [22]. These findings suggest that the decrease in production of 1,25-$(OH)_2D_3$ in THP-1 cells might result from an increase in the expression of 24-hydroxylase but not from a decrease in the expression of 1α-hydroxylase.

In renal cells, 1,25-$(OH)_2D_3$ suppressed the expression of 1α-hydroxylase gene at the transcriptional level. Murayama et al demonstrated that 10^{-8} mol/L 1,25-$(OH)_2D_3$ suppress expression of 1α-hydroxylase mRNA by about 80% in mouse renal proximal tubule cells, and found a negative regulatory region for 1,25-$(OH)_2D_3$ located at around −0.5 kb in the human 1α-hydroxylase gene [33]. However, we and other groups observed that porcine renal proximal tubule LLC-PK_1 cells lack the 1,25-$(OH)_2D_3$ transrepression system of the 1α-hydroxylase gene despite the presence of the 1,25-$(OH)_2D_3$ transactivation system of 24-hydroxylase gene [34, 35]. These findings suggest that the 1,25-$(OH)_2D_3$ transactivation system is conserved, whereas the 1,25-$(OH)_2D_3$ transrepression system was lost in THP-1 and LLC-PK_1 cells.

The majority of studies have examined the activation of gene expression by 1,25-$(OH)_2D_3$. Vitamin D_3 response elements (VDREs) consist of two direct repeats, comprised of the consensus sequence AGGTCA, which

are separated by a 3 bp spacer [36]. However, VDREs have also been implicated in negative regulation of gene expression in the human PTH, chicken PTH, human PTH-related peptide, rat PTH-related peptide, human interleukin-2, and the rat bone sialoprotein genes [37–42]. The chicken PTH-negative VDRE is similar to the consensus positive VDRE, except that the last two bases (CA) in the 3′-half element are replaced by other nucleotides (GT). When altering the 3′-terminal bases (GT) back to the consensus CA, the chicken PTH VDRE reverted from a negative to a positive VDRE [43]. In the case of the human PTH gene, the negative VDRE contains a sequence homologous to only one of the two hexameric DNA sequences that form the positive VDRE. It was shown that VDR, but not retinoic acid X receptor (RXR), was involved in this process, and the presence of another unknown partner protein(s) of VDR was proposed [44]. These observations led us to speculate that macrophages lack some nuclear proteins binding to the negative VDREs, or some nuclear receptor coactivators that could mediate the diverse response of 1,25-$(OH)_2D_3$.

Macrophage 1α-hydroxylase that causes hypercalcemia in sarcoidosis has been attracting wide interest. The regulation of macrophage 1α-hydroxylase has generally been studied by measuring enzymatic activity, but the net production of 1,25-$(OH)_2D_3$ is determined by both 1α-hydroxylase-mediated activation and 24-hydroxylase-mediated inactivation. Since it is difficult to measure 1α-hydroxylase activity separate from the effect of other hydroxylases, the regulation of macrophage 1α-hydroxylase has not been fully elucidated. The molecular cloning of 1α-hydroxylase enabled us to investigate the regulation at a molecular level. We have shown, to our knowledge for the first time, that macrophage 1α-hydroxylase is the same gene product as the renal enzyme, and we provided direct evidence that the sensitivity to interferon-γ and resistance to 1,25-$(OH)_2D_3$ take place at the mRNA level.

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